

# Parsimonious immune-response endotypes and global outcome in patients with traumatic brain injury



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## Summary

**Background** The inflammatory response in patients with traumatic brain injury (TBI) offers opportunities for stratification and intervention. Previous unselected approaches to immunomodulation in patients with TBI have not improved patient outcomes.

**Methods** Serum and plasma samples from two prospective, multi-centre observational studies of patients with TBI were used to discover (Collaborative European NeuroTrauma Effectiveness Research [CENTER-TBI], Europe) and validate (Transforming Research and Clinical Knowledge in Traumatic Brain Injury [TRACK-TBI] Pilot, USA) individual variations in the immune response using a multiplex panel of 30 inflammatory mediators. Mediators that were associated with unfavourable outcomes (Glasgow outcome score-extended [GOS-E]  $\leq 4$ ) were used for hierarchical clustering to identify patients with similar signatures.

**Findings** Two clusters were identified in both the discovery and validation cohorts, termed early-inflammatory and pauci-inflammatory. The early-inflammatory phenotype had higher concentrations of interleukin-6 (IL-6), IL-15, and monocyte chemoattractant protein 1 (MCP1). Patients with the early-inflammatory phenotype were older and more likely to have an unfavourable GOS-E at 6 months. There were no differences in the baseline injury severity scores between patients in each phenotype. A combined IL-15 and MCP1 signature identified patients with the early-inflammatory phenotype in both cohorts. Inflammatory processes mediated outcomes in older patients with moderate-severe TBI.

**Interpretation** Our findings offer a precision medicine approach for future clinical trials of immunomodulation in patients with TBI, by using inflammatory signatures to stratify patients.

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**Keywords:** Traumatic brain injury; Stratified medicine; Inflammation; Clustering

### Research in context

#### Evidence before this study

Activation of the immune response following traumatic brain injury (TBI) has been shown to be associated with unfavourable neurological outcomes in both patients and animal models of TBI. High circulating concentrations of various immune mediator proteins (cytokines and chemokines) following TBI are widely reported with evidence of innate immune cell activation and migration to injured brain tissue. In patients with repeated head injury that develop a leukoencephalopathy a chronic neuroinflammatory process is apparent and there is concern that an excessive, early immune response may lead to prolonged neuroinflammation and unfavourable neurological outcomes. Attempts to address this immune response in a randomised controlled trial of corticosteroids did not demonstrate benefit to patients suggesting that a stratified medicine approach is required to identify the patient sub-populations who are most likely to benefit from immune modulating therapies.

#### Added value of this study

We measured circulating concentrations of immune mediators in patients with moderate-severe TBI recruited to two distinct, multi-centre observational studies. We identified two groups of patients in both studies who had similar

signatures of inflammation that were consistently related to functional neurological outcomes. These patients had raised concentrations of at least two circulating cytokines: interleukin (IL) 15 and monocyte chemoattractant protein (MCP) 1, suggesting an early-inflammatory response to TBI. We showed that these patients were more likely to be older and that this pattern of inflammation was not associated with injuries to other parts of the body.

#### Implications of all the available evidence

The inflammatory response to TBI has repeatedly been shown to associate with unfavourable patient outcomes but it has been difficult to translate this into therapeutic interventions that might benefit patients. In addition, it is essential that other confounding variables are accounted in these patients who may have multiple causes for an inflammatory surge following injury. We identified a parsimonious signature consisting of two circulating immune mediators (IL-15 and MCP1), that can be used to identify patients who are at greater risk of poor functional neurological outcomes following moderate-severe TBI. Our findings are consistent with the wider biological understanding of the immune response to TBI and may facilitate stratification of patients for future randomised controlled trials.

### Introduction

Traumatic brain injury (TBI) is a major cause of death and disability, and while improvements in supportive care have led to a reduction in mortality, the proportion of patients making a good recovery has not improved to the same degree.<sup>1,2</sup> This highlights the urgent need for therapeutic interventions that can improve recovery trajectory and not simply improve survival. Neuroinflammation is recognised as a key mediator of secondary brain injury,<sup>3</sup> which is brain damage attributable to downstream processes triggered by the initial physical injury, and in some patients, is associated with chronic inflammation and accelerated neurodegeneration affecting their long-term outcomes.<sup>3,4</sup> Neuroinflammation therefore represents a therapeutic target that may be addressed by re-purposing current, widely available medications such as monoclonal antibodies that affect cytokine activity (e.g., tocilizumab), small molecules (e.g., granulocyte colony stimulating factor), mesenchymal stem cells and other anti-inflammatory compounds (progestagens, glucocorticoids).<sup>5–9</sup> However, immunomodulation in critically ill patients risks increasing susceptibility to potentially life-threatening infections. While recent experience with immunomodulators in COVID-19 suggested that they were safer than originally feared, patients treated with these agents

continued to show an excess of some complications, such as fungal infections.<sup>10</sup> Perhaps even more importantly, the CRASH trial showed that treatment of all patients with an anti-inflammatory intervention (high-dose corticosteroids) did not provide benefit, and resulted in harm.<sup>11</sup> It is therefore important to identify patients who are most likely to benefit from these treatments.

Attempts to discover distinct mechanistic subtypes (endotypes) in other critical illnesses such as acute respiratory distress syndrome (ARDS) have highlighted inflammatory sub-phenotypes as important categorisations, with patients predisposed towards a “hyper-inflammatory” phenotype experiencing worse outcomes.<sup>12</sup>

A seminal study by Brankenridge et al. established that multiomic signatures were associated with survival in patients with polytrauma in the PAMPer study of fresh thawed plasma in major trauma.<sup>13</sup> These findings were recapitulated in a subgroup of patients with TBI in this cohort<sup>14,15</sup> and validated in an independent cohort.<sup>16</sup> However, these endotypes were based on a six-layer classification process, aggregating components from several biomarker classes to determine endotype membership. Despite offering comprehensive phenotyping of patients, it was not readily implementable for patient

selection for clinical trials targeting immune modulation. Further, mortality was the only endpoint assessed, which is a limitation as in acute neurological conditions the impact of endotypes on functional TBI outcomes is crucial.

We sought to address these issues by undertaking an analysis of the impact of the inflammatory response in a cohort of patients with moderate to severe TBI from the Collaborative European NeuroTrauma Effectiveness Research (CENTER-TBI) study.<sup>17</sup> Identification of a parsimonious set of mediators in patients with TBI might allow for classification for future clinical trials, exploration of networks that might underpin these responses, and establishing associations with not just mortality, but the full range of the Glasgow Outcome Score-Extended (GOS-E).<sup>18</sup> Further, we sought to validate our results in a corresponding independent cohort from the Transforming Research and Clinical Knowledge in Traumatic Brain Injury (TRACK-TBI Pilot) study.<sup>19</sup>

## Methods

### Study populations

The discovery cohort was sourced from the CENTER-TBI Core study, conducted at 65 clinical sites between 2014 and 2017 (Clinical-Trials.gov: NCT02210221). The study protocol, sample size calculations and clinical data have been previously published.<sup>17</sup> Patients presenting to a study centre within 24 h of injury and scheduled for CT scanning were enrolled and stratified by care path: emergency department, hospital ward or intensive care unit (ICU) admission. The validation cohort was composed of patients recruited to the prospective, pilot, multi-centre observational study Transforming Research and Clinical Knowledge in Traumatic Brain Injury (TRACK-TBI Pilot) study, which was conducted at three Level 1 trauma centres in the United States between 2010 and 2013 (Clinical-Trials.gov: NCT01565551).<sup>20</sup>

Baseline clinical features including patient demographic characteristics, clinical presentation, and CT brain imaging were recorded. Data on sex (but not gender) was gathered at the point of enrolment, and obtained from the participant, next of kin where available, or inferred by biological characteristics. We included in our analyses clinical characteristics that are validated predictors of patient outcomes in TBI including Glasgow Coma Scale (GCS) at presentation, pupillary reactivity, prehospital or emergency room (ER) hypoxia, prehospital or ER hypotension and any major extracranial injury (MEI), which was defined as an Abbreviated Injury Score (AIS)  $\geq 3$  in any non-cranial region.<sup>21</sup> CT brain characteristics were graded using the Marshall classification.<sup>22</sup>

The inclusion and exclusion criteria for patients enrolled in the CENTER-TBI and TRACK-TBI Pilot

studies have previously been published and are included in [Supplementary Table S1](#). The only additional inclusion criteria for our analysis were patients who had suffered moderate-severe TBI, defined as admission GCS  $\leq 12$ , and who had additional samples on both the day of admission and on day 2–3 following admission available for inflammatory profiling. Only admission day samples were available in the validation cohort. The study is reported in accordance with STROBE recommendations.

### Inflammatory-mediator and brain injury biomarker profiling

In the discovery cohort samples were collected using gel-separator tubes for serum and centrifuged within 60 min of sampling. The serum was aliquoted and stored at  $-80^{\circ}\text{C}$ . In the validation cohort, blood was sampled within 24 h of injury in EDTA tubes, which were centrifuged with plasma aliquoted and stored at  $-80^{\circ}\text{C}$  within 60 min. Patients with moderate-severe TBI (GCS  $\leq 12$ ) in the discovery cohort who had samples available on the day of admission and on day 2–3 after admission were selected for inflammatory profiling.

Inflammatory mediator concentrations were measured in multiplex with Mesoscale Discovery (MSD, Rockville, MD, USA) V-plex using three standard, human 10-plex panels; pro-inflammatory –1, cytokine-1 and chemokine-1 in both cohorts (catalogue nos. K15049D, K1505D, K15047D). The cytokines and chemokines quantified were Eotaxin-1, Eotaxin-3, granulocyte-macrophage colony stimulating factor (GM-CSF), interferon gamma (IFN $\gamma$ ), interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-13, IL-15, IL-16, IL-17a, monocyte chemoattractant protein (MCP) 1, MCP4, macrophage-derived chemokine (MDC), macrophage inflammatory protein (MIP)-1 $\alpha$ , MIP-1 $\beta$ , thymocyte activation-regulated chemokine (TARC) tumour necrosis factor (TNF)  $\alpha$ , TNF $\beta$  and vascular endothelial growth factor (VEGF).

For the discovery cohort, 10% of the samples were measured in duplicate and concentrations were calibrated using standard curves and with duplicate control serum on each plate. Intra- and inter-plate coefficients of variation are included in the [Supplementary Table S2](#). Samples from the discovery and validation cohorts were measured in the different laboratories (Discovery Cohort at Centre Médical Universitaire, Genève, Switzerland; Validation Cohort at the University of Florida, Gainesville, USA) but with the same immunoassay platform (MSD) in accordance with the manufacturer's specifications.

Inflammatory profiling of patients with TBI in the validation cohort of all severities (mild, moderate, severe) and comparison with orthopaedic controls and healthy controls has previously been reported.<sup>23</sup>

Patients in the discovery cohort had data available for six brain injury biomarkers: glial fibrillary acidic protein

(GFAP), neurofilament light (NfL), total Tau (tTau), ubiquitin c-terminal hydroxylase L1 (UCH-L1), S100 calcium binding protein B (S100B) and neuron-specific enolase (NSE). GFAP, NfL, tTau and UCH-L1 were quantified using a Human Neurology 4-Plex B assay run on the Quanterix Simoa SR-X platform (Quanterix Corp., Lexington, MA) at the University of Florida (Gainesville, Florida); S100B and NSE were analysed using an electrochemiluminescence immunoassay (Elecys S100 and Elecys NSE assays) run on the e 602 module of Cobas 8000 modular analyser (Roche Diagnostics, Mannheim, Germany) at the University of Pécs (Pécs, Hungary). The association between these biomarkers, clinical features and outcomes has previously been reported.<sup>24,25</sup>

### Statistics

Outcomes were measured using the Glasgow Outcome Score-Extended (GOS-E) which ranges from 1 (death) to 8 (full recovery). We designated unfavourable outcomes if patients had a GOS-E at 6 months less than upper severe disability (4) in patients with moderate-severe TBI. To make statistical comparisons between groups, we used non-parametric tests (Wilcoxon-Rank sum) for continuous, non-normally distributed variables, t-tests for normally distributed variables, and chi-squared tests for categorical variables. Variable distributions were checked for normality using Shapiro–Wilks test. The Benjamini-Hochberg procedure to correct for false discovery rates (FDR) was used for correction of *p*-values. Missing data were not imputed. The *p*-value threshold for significance was < 0.05. All analysis was conducted using R version 4.2.

### Network analysis of inflammatory mediators

We used Metacore™ (version 21.3, Clarivate analytics, Philadelphia, USA) for network analysis of inflammatory mediators to identify the associated signalling proteins and transcription factors that might relate to different patient outcomes. The shortest paths network algorithm option was used to generate protein signalling networks, with maximum number of steps in the path set to two.

### Cluster assignment and baseline clinical features

To identify which patients had signatures consistent with different inflammatory pathways, we used hierarchical clustering with Ward linkage and Euclidean distance of the mediators that were associated with outcomes. The optimum number of clusters was selected using the silhouette method. The same clustering strategy was used in both cohorts. Cluster assignments were visualised using principal component plots.

We compared the baseline clinical features, inflammatory mediator concentrations and patient outcomes in each cluster using odds ratios and correlation coefficients. The correlations between mediators within in

each cluster were calculated using Pearson's method and visualised using heatmaps.

### Cluster prediction and homogeneity across cohorts

To assess our clustering approach, we fitted a logistic regression model with an elastic net penalisation to our cluster assignments in the discovery cohort.<sup>26</sup> This approach allowed us to fit a classifier model that performed well despite the high degree of correlation between predictor variables, by balancing L1 (lasso) and L2 (ridge) penalties. The alpha and lambda values for the model were optimised using leave-one-out cross-validation.

We checked for homogeneity between the identified clusters in two ways. First, we used our model, fitted on the discovery cohort, to predict clusters in the validation cohort. Classifier performance was assessed by the comparison of predicted cluster with the cluster labels from hierarchical clustering. Secondly, we used Mantel's permutation test on the inter-biomarker correlations within each cluster. This test calculated the correlations between two distance matrices and assessed the null hypothesis that two distance matrices were not correlated.

The best-performing biomarker for cluster prediction in each cohort was determined using the calculated lambda values and internal cross-validation.

### Identification of parsimonious signatures across both cohorts

We identified the most consistent biomarkers across cohorts with logistic regression that was restricted to the best performing variables identified by our elastic net model. Models were trained to predict cluster assignment in the discovery cohort and tested on the validation cohort. Model performance was evaluated using area under receiver operating characteristic curve (AUROC), Akaike information criterion (AIC), likelihood ratio test, and calibration was assessed using estimates of intercept and slope of the calibration curve following 1000 bootstrapped repetitions.

### Cluster stability

In the discovery cohort, additional samples taken on day 2 or day 3 after admission, were also available. The inflammatory mediator values from these later samples were clustered independently. Cluster stability was assessed using the adjusted Rand index. We assessed the association between cluster status at admission and on day 2/3, to explore whether patients had moved or remained in each cluster and related cluster membership at each sampling time to GOS-E at 6 months. Transitions between clusters were demonstrated by Sankey diagrams.

### Mediation analysis

To determine whether the association between TBI and patient outcomes was mediated by inflammatory

processes we used a mediation analysis approach.<sup>27</sup> Briefly, mediation analysis is a causal inference method that calculates the relative influence of intermediate variables on a given outcome, the relationships between which are described by a directed acyclic graph (DAG).

In our causal model we used GFAP levels as a convenient continuous marker of the overall burden of brain injury. GFAP has been consistently shown to be associated with outcomes of brain injury and peaks early after injury.<sup>24,28–30</sup> GFAP samples were collected at the same time as the inflammatory mediators. We used the first principal component of the inflammatory mediators that were significantly associated with unfavourable outcomes as the mediator variable. We also accounted for age and major extracranial injury (MEI) in our model. These variables are associated with TBI outcomes<sup>21</sup> and were likely to confound both outcome and activation of inflammatory pathways. Prior to estimation of predictor effects, the independence of predictor variables was assessed using d-separation tests and the linearity assumption between predictors and outcomes for a logistic regression model was assessed using component-residual plots. Estimates of the direct and indirect causal paths, were calculated using the *brms* package in R and presented with 95% confidence intervals.

To ensure that we were not unmasking spurious causation, we calculated the correlation coefficients between clinical features and the first principal component of inflammatory mediators associated with unfavourable outcomes to check for collinearity.

### Relationship between brain injury biomarkers and inflammatory mediators

To demonstrate the relationship between circulating brain injury biomarkers and inflammatory mediators in patients with moderate-severe TBI, we visualised their relative loadings using principal component analysis plots. We determined whether processes described by each set of protein biomarkers were orthogonal by calculating the dot product of the loading vectors, weighted by the explained variance of each principal component. A dot product equal to 0 would imply that these data were completely orthogonal. To check our results, we conducted a permutation test using 10,000 random draws and calculated whether the distribution of permuted dot products was likely to include 0.

### Ethics

The CENTER-TBI study (EC grant 602,150, NCT02210221) has been conducted in accordance with all relevant laws of the EU if directly applicable or of direct effect and all relevant laws of the country where the Recruiting sites were located, including but not limited to, the relevant privacy and data protection laws and regulations (the “Privacy Law”), the relevant laws

and regulations on the use of human materials, and all relevant guidance relating to clinical studies from time to time in force including, but not limited to, the ICH Harmonised Tripartite Guideline for Good Clinical Practice (CPMP/ICH/135/95) (“ICH GCP”) and the World Medical Association Declaration of Helsinki entitled “Ethical Principles for Medical Research Involving Human Subjects”. Informed Consent by the patients and/or the legal representative/next of kin was obtained, accordingly to the local legislations, for all patients recruited in the Core Dataset of CENTER-TBI and documented in the e-CRF.

Ethical approval was obtained for each recruiting sites. The list of sites, Ethical Committees, approval numbers and approval dates can be found at <https://www.center-tbi.eu/project/ethical-approval> and <https://www.center-tbi.eu/files/project/IRB-overview-v2.pdf>.

The TRACK-TBI Pilot study (NCT01565551) was conducted following institutional review board (IRB) approval at each participating site: San Francisco General Hospital (SFGH), University of Pittsburgh Medical Center (UPMC), and University Medical Center Brackenridge (UMCB) in Austin, Texas. The overall study received approval from the IRB at the University of California, San Francisco (UCSF; Protocol No.: 10-00111). Informed consent was obtained before enrolment. For patients unable to provide consent because of the severity of their injury, consent was obtained from their legally authorised representative or surrogate next of kin. Patients were reconsented, if cognitively able, during their clinical care and/or follow-up time points regarding continuation in study participation.

### Role of funders

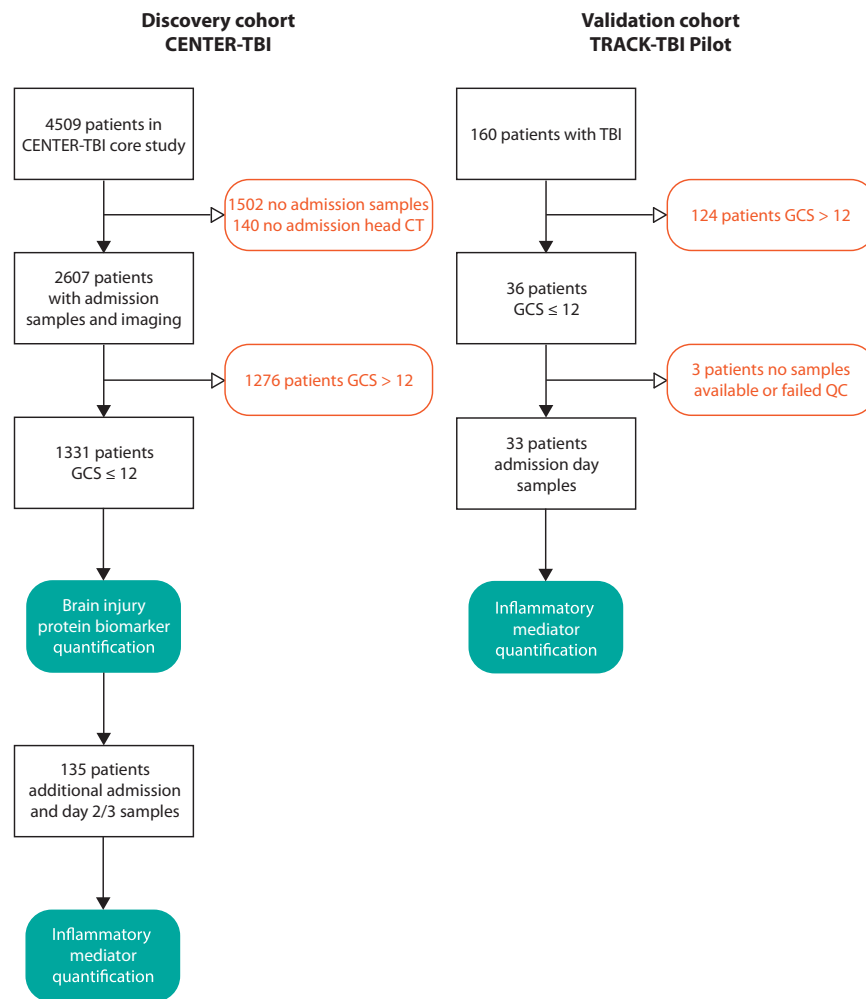
The funders had no role in the study design, collection, analysis and interpretation of data, nor in the writing of the report or in publication decisions.

## Results

### Patient characteristics

The number of patients with moderate-severe TBI with adequate samples for inflammatory profiling was 135 in the discovery cohort and 33 in the validation cohort (Fig. 1). The patients who had inflammatory profiling in the discovery cohort were of similar baseline characteristics to the wider CENTER-TBI study with respect to age, etiology, baseline GCS and major extracranial injury (MEI). However, the patients selected for inflammatory profiling were more likely to be male and there were differences in the Marshall score of CT images of these patients compared with patients in the wider CENTER-TBI study with moderate-severe TBI (Supplementary Table S3).

Baseline patient characteristics of patients that had inflammatory profiling in the discovery and validation cohorts are presented in Table 1. The commonest



**Fig. 1:** Flow diagram demonstrating the number of samples used for analysis in each cohort (CENTER-TBI  $n = 135$ , TRACK-TBI  $n = 33$ ).

causes of injury were road traffic accidents and falls in both cohorts. The median age of patients in the CENTER-TBI cohort was 46 years (IQR 28–62) and in the TRACK-TBI Pilot cohort was 53 year (IQR 25–69). There were notable difference in several baseline features between cohorts including proportion of male patients, initial motor GCS and Marshall CT scores (Table 1).

#### Divergent inflammatory processes at the time of recruitment in patients with TBI are associated with different outcomes

Higher serum concentrations of IL-6, IL-8, IL-15, IL-16, and MCP1 at the time of recruitment were associated with unfavourable outcomes in patients in the discovery cohort (Fig. 2). Network analysis of the inflammatory mediators that were significantly associated with unfavourable outcomes in the discovery cohort identified IL-6, IL-8, IL-15, and MCP1 as belonging to an

interaction network closely associated with the transcription factor signal transducer and activation of transcription (STAT) 3 (Fig. 3a). In the validation cohort, while IL-8 and IL-16 were no different between outcome groups, IL-6, IL-15, and MCP1 were higher in the group that had unfavourable outcomes. However, these differences did not achieve significance (Supplementary Fig. S1), which we attributed to the small sample size.

#### Clustering identified patients with inflammatory signatures that were associated with outcomes

Hierarchical clustering of patients using the five inflammatory mediators associated with unfavourable outcomes identified two optimum clusters (Fig. 3b) using the silhouette method (Supplementary Fig. S2). One cluster, which was larger and consisted of 106 patients (79%), had consistently higher concentrations of these five mediators compared to the other cluster. Patients in this larger cluster also had significantly higher

concentrations of other inflammatory mediators that were not included by the data-driven model to generate the clusters, including IP10, MCP4, MDC, MIP-1 $\alpha$ , MIP-1 $\beta$ , TARC, IFN $\gamma$ , and IL-10 (Table 2, Supplementary Fig. S3). Patients in the smaller cluster had significantly higher concentrations of IL-13 and TNF $\beta$ . Based on these findings we termed these clusters as “early-inflammatory” and “pauci-inflammatory.”

Using the same five inflammatory mediators and clustering methods, we also identified two clusters in the validation cohort (Fig. 3b). The larger cluster consisted of 24 (69%) patients with higher plasma concentrations of IL-15, IL-6, and MCP1, but not IL-8 and IL-16. Similar to the discovery cohort, patients in the larger validation cohort cluster had significantly higher concentrations of IL-10, TNF $\alpha$ , and MIP-1 $\alpha$  (Table 2, Supplementary Fig. S4). Clusters from both cohorts were well separated in the principal component space (Supplementary Fig. S5). Given the similar biological features of the identified clusters in the discovery and validation cohort (Fig. 3b) we labelled these subgroups in the same manner.

In the discovery cohort, 67% of patients assigned to the early-inflammatory cluster had an unfavourable outcome, compared to 36% in the pauci-inflammatory cluster (OR 3.72 95% CI 1.53–8.99;  $p = 0.0036$ ). In the validation cohort 59% of patients assigned to the early-inflammatory cluster, compared with 12% assigned to the pauci-inflammatory cluster, had an unfavourable outcome (OR = 11.38 95% CI 1.17–110.42;  $p = 0.036$ ; Table 3). Although the GOS-E were dichotomised for our statistical analysis, we found that greater proportions of patients with the pauci-inflammatory cluster had GOS-E in the lower disability categories in both cohorts (Fig. 3c).

The five inflammatory mediators that defined the clusters were significantly positively correlated with each other in the discovery cohort (Supplementary Fig. S7). A similar positive correlation between cluster cytokine members was found in the validation cohort, except for IL-16, which was only positively correlated with IL-8, but negatively correlated with the other members (Supplementary Fig. S6b).

### Clinical characterisation of patients in inflammatory clusters

Assignment of patients to clusters facilitated the exploration of the clinical features of patients. In the discovery cohort, early-inflammatory patients were more likely to be older (median age 49 vs 32,  $r = 0.22$  [95% CI 0.055–0.38;  $p = 0.0096$ ]). There were no differences in baseline GCS, or other early adverse features associated with unfavourable outcomes in TBI such as early hypoxaemia and hypotension (Table 3). Importantly there was no association between cluster assignment and injury severity score (ISS) or MEI, nor was there an association with Marshall scores of CT brain images.

|                                      | Discovery,<br>N = 135 | Validation,<br>N = 33 | p value |
|--------------------------------------|-----------------------|-----------------------|---------|
| Sex, n (%)                           |                       |                       | 0.0047  |
| Female                               | 25 (19)               | 14 (42)               |         |
| Male                                 | 110 (81)              | 19 (58)               |         |
| Age (years), Median (IQR)            | 46 (28–62)            | 53 (25–69)            | 0.49    |
| Initial GCS, Median (IQR)            | 6 (3–8)               | 4 (3–9)               | 0.76    |
| Unknown                              | 0                     | 1                     |         |
| Moderate TBI, n (%)                  | 26 (19)               | 9 (28)                | 0.27    |
| Severe TBI, n (%)                    | 109 (81)              | 23 (72)               | 0.27    |
| Initial motor GCS, Median (IQR)      | 3 (1–5)               | 5 (2–6)               | 0.0022  |
| Baseline pupillary reactivity, n (%) |                       |                       | 0.13    |
| Both pupils unreactive               | 26 (21)               | 7 (23)                |         |
| Both reactive                        | 85 (67)               | 23 (77)               |         |
| One pupil unreactive                 | 15 (12)               | 0                     |         |
| Unknown                              | 9                     | 3                     |         |
| Pre-hospital/ED hypotension, n (%)   |                       |                       | 0.71    |
| No                                   | 100 (86)              | 29 (88)               |         |
| Suspected                            | 4 (3.4)               | 0                     |         |
| Yes                                  | 12 (10)               | 4 (12)                |         |
| Unknown                              | 19                    | 0                     |         |
| Pre-hospital/ED hypoxia, n (%)       |                       |                       | 0.55    |
| No                                   | 102 (88)              | 31 (94)               |         |
| Suspected                            | 6 (5.2)               | 0 (0)                 |         |
| Yes                                  | 8 (6.9)               | 2 (6)                 |         |
| Unknown                              | 19                    | 0                     |         |
| Marshall CT score                    |                       |                       | <0.0001 |
| I                                    | 5                     | 7                     |         |
| II–IV                                | 54                    | 20                    |         |
| V–VI                                 | 70                    | 7                     |         |
| Unknown                              | 6                     | 0                     |         |
| Injury severity score, Median (IQR)  | 34 (25–50)            | 25 (16–29)            | <0.0001 |
| Major extracranial injury, n (%)     | 86 (64)               | 10 (37)               | 0.012   |
| Unknown                              | 0                     | 6                     |         |

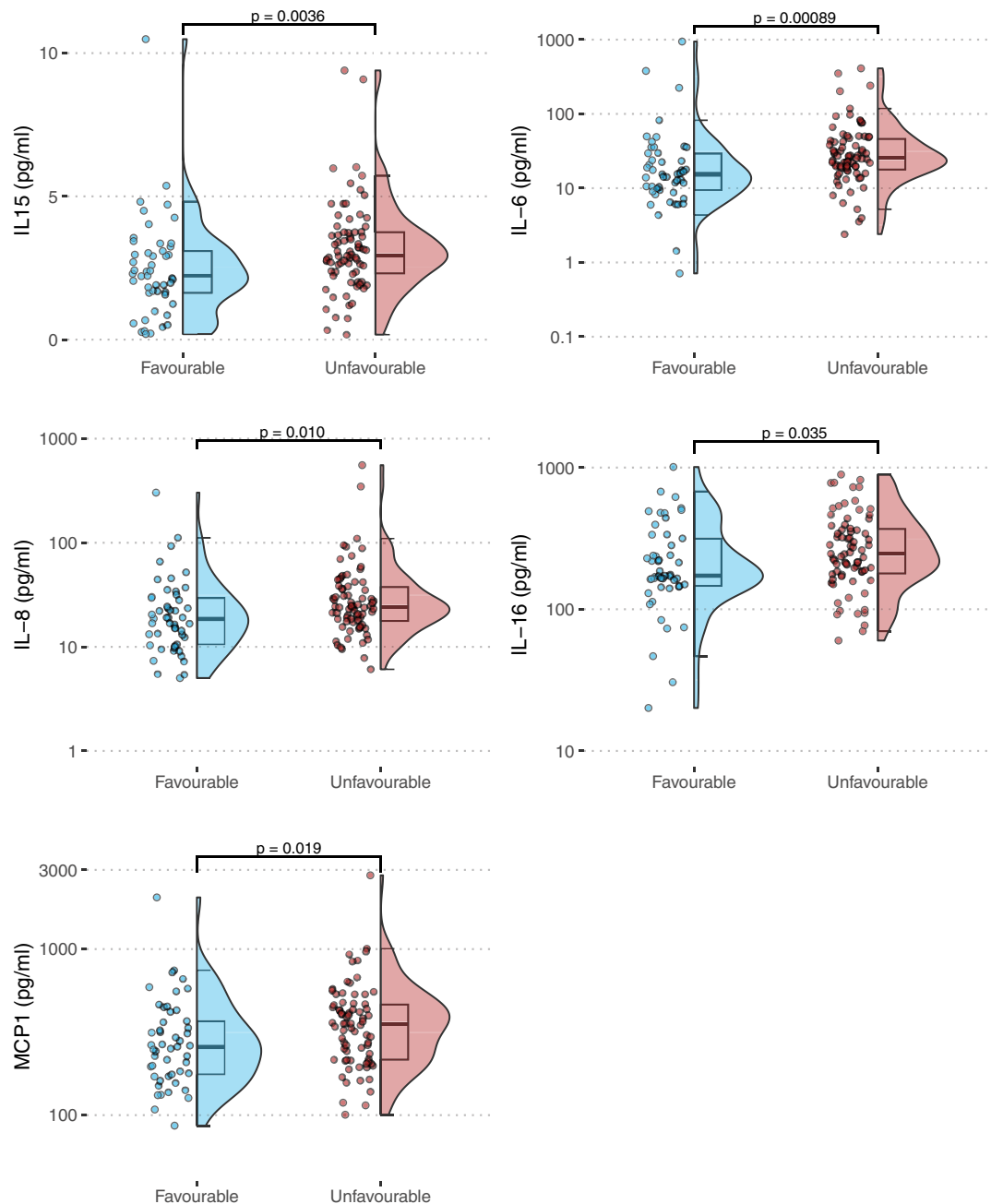
GCS: Glasgow coma score, ED: emergency department.

**Table 1:** Baseline features of patients in both cohorts with moderate-severe traumatic brain injury.

In the validation cohort, patients assigned to the early-inflammatory cohort were older than patients in the pauci-inflammatory group (median age 62 vs 24,  $r = 0.52$  [95% CI 0.22–0.73];  $p = 0.0017$ ). Similar to the discovery cohort, there were no differences in ISS, MEI, early hypoxaemia or early hypotension between clusters in the validation cohort. However, there were differences in initial GCS, which was lower for patients in the early-inflammatory group compared with patients in the pauci-inflammatory group (median = 3 vs 8,  $r = -0.37$  [95% CI = -0.63 to -0.022];  $p = 0.038$ , Table 3).

### Homogeneity between early-inflammatory clusters in each cohort

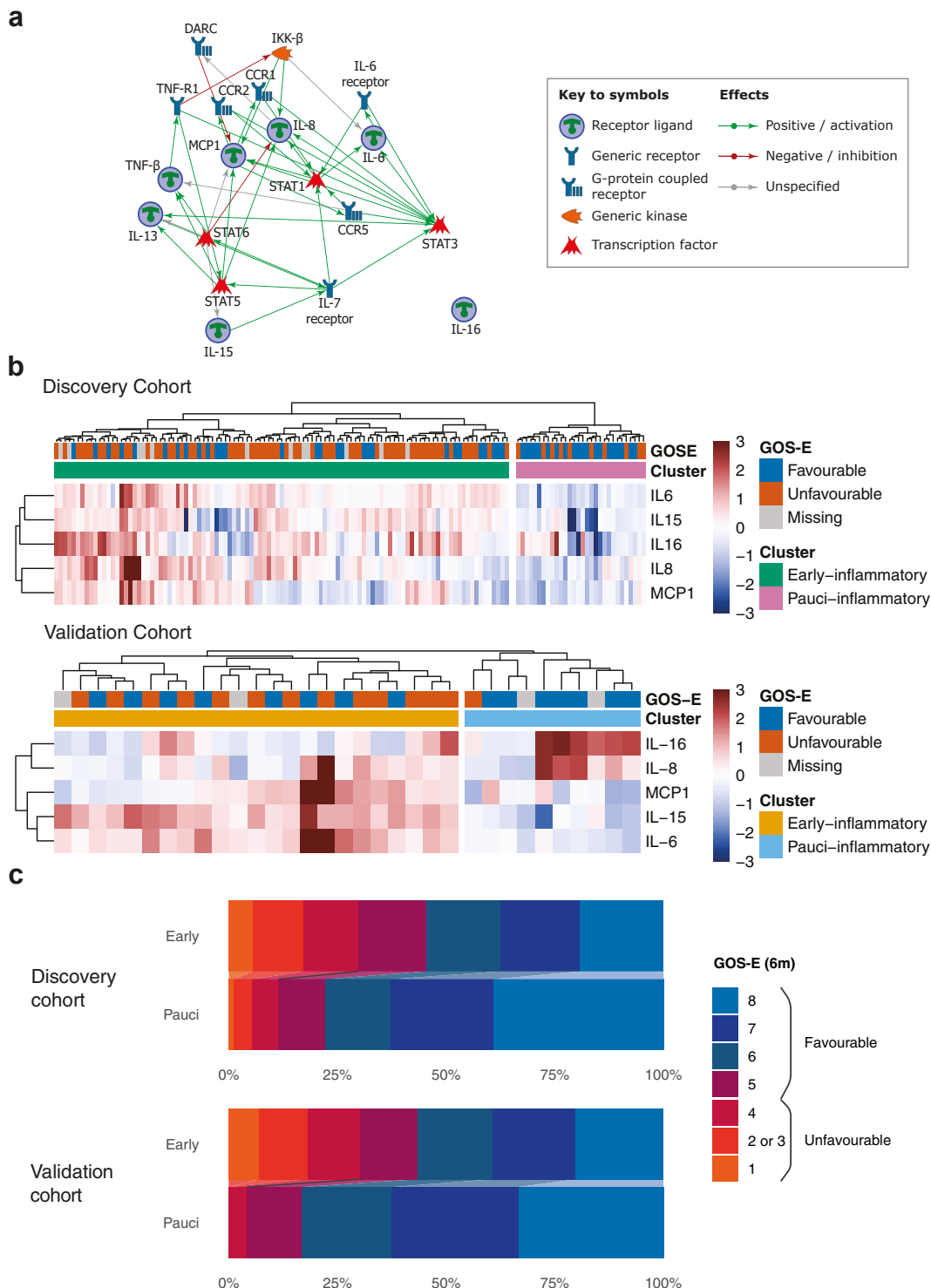
We fitted a logistic regression model with elastic net penalisation for the discovery cohort clusters, using leave-one-out cross-validation, which calculated the



**Fig. 2:** The concentrations and distributions of inflammatory mediators in patients with moderate-severe TBI, at the time of admission to hospital that were significantly associated with unfavourable outcomes in the discovery cohort (CENTER-TBI,  $n = 135$ , single samples, no biological or technical replicates). Points are individual patient values. The distributions of the data are demonstrated by the box plot (box: median, IQR; whiskers:  $1.5 \times \text{IQR}$ ) and violin plots (kernel density estimate).

optimum alpha and lambda values of 0.1 and 0.0007 respectively. When testing cluster prediction on the validation cohort, the AUROC was 0.90 (95% CI 0.83–0.97). The Mantel permutation test determined that the correlations between inflammatory mediators within each early-inflammatory cluster were consistent

for the early-inflammatory clusters in both cohorts ( $r = 0.75$ ,  $p = 0.043$ , Mantel's test). However, this was not the case for the observed correlations between mediators when comparing the pauci-inflammatory clusters with each other ( $r = -0.14$ ,  $p = 0.53$ , Mantel's test).



**Fig. 3:** The inflammatory mediators associated with outcomes in TBI are related to divergent inflammatory processes. **(a)** Network analysis of the cytokines associated with outcomes using Metacore software which identified STAT1 and STAT3 as being central transcription factors closely related to IL-6, IL-8, IL-15 and MCP1. **(b)** Heatmaps showing the scaled values of inflammatory mediators in each cluster, alongside patient outcomes, in both cohorts (CENTER-TBI  $n = 135$ , TRACK-TBI  $n = 33$ ). There was a consistent higher concentration of IL-15 in both early-

### IL-15 and MCP1 formed the most parsimonious signature across both cohorts

In the discovery cohort, we identified three multi-variate models that performed well for identifying cluster assignment: IL-15 & MCP1; IL-15 & IL-6; and IL-15, IL-6 & MCP1. These combinations of variables had similar performance characteristics for predicting cluster assignment in the validation data ([Supplementary Table S4](#)). There was no significant difference between the IL-15 & MCP1 model and the three variables model (IL-15, MCP1, IL-6) when using the likelihood ratio test. The combination of IL-15 and MCP1 was chosen as the most parsimonious variable for cluster prediction due to the lower AIC (73.2) and higher AUROC (0.94 [95% CI 0.90–0.99]; [Supplementary Table S4](#)). The calibration curve for this model on predicting outcomes in the validation cohort demonstrated in [Supplementary Fig. S7](#).

### Inflammatory status at day 2/3 was associated with patient outcome

Clustering of patients at each sampling time in the discovery cohort was carried out independently, and the transitions between sub-phenotypes were observed ([Supplementary Fig. S8](#)). Altogether 23 of the 135 patients (17%) transitioned from one cluster to another. The adjusted Rand index calculated cluster stability as 0.37 which was consistent with moderate stability of cluster assignment over this short time window. Patients who were initially in the early-inflammatory subgroup were more likely to have an unfavourable outcome (OR 3.74, 95% CI 1.35–10.37;  $p = 0.011$ ), irrespective of whether they transitioned or not to a different cluster on day 2/3.

### Mediation analysis supported an intermediate causal path for inflammatory processes between GFAP and GOS-E at 6 months

The causal model we used to estimate the mediated effect of inflammation is shown in [Fig. 4](#). Prior to estimating effects for variables in our model we demonstrated that predictor variables satisfied the linearity assumption ([Supplementary Fig. S9](#)).

The direct causal mediated path between GFAP and outcome, whilst accounting for major extracranial injury and age, was significantly associated with unfavourable outcomes (0.99, 95% CI 0.49–1.49). The first principal component (PC1) of five inflammatory markers (IL-6, IL-8, IL-15, IL-16, MCP1) was significantly associated with unfavourable outcomes (0.56, 95% CI 0.24–0.92) and the adjusted causal mediated effect was significant

(0.11, 95% CI 0.010–0.27) ([Supplementary Table S5](#)). If we excluded age as a confounder then the adjusted causal-mediated effect through PC1 was not significant (0.16, 95% CI –0.030 to 0.48, [Supplementary Table S6](#)). This suggested that the association between the severity of TBI, which we estimated using GFAP, and patient outcomes, was mediated by the host inflammatory response when accounting for both age and MEI. The standardised estimates for causal paths are shown in [Supplementary Fig. S10](#).

A direct exploration of the relationship between age and inflammation showed that the first principal component of the inflammatory response was not directly and significantly correlated with age. This was the case in both the discovery ( $r = 0.023$ , 95% CI –0.15 to 0.19;  $p = 0.79$ ) and validation cohorts ( $r = -0.28$ , 95% CI –0.59 to 0.069;  $p = 0.11$ ) and suggested that inflammation and age were not collinear in patients with moderate-severe TBI.

### Brain injury biomarkers and inflammatory mediators are orthogonal in the PCA space

Inflammatory mediators and brain injury markers in the discovery cohort were co-linear with respect to themselves but relatively orthogonal with respect to each other and these differences were most apparent in the second principal component ([Fig. 5](#)). Permutation analysis calculated the mean dot product was –0.019 (95% CI –0.074 to +0.035), suggesting that it was not significantly different from zero and these data were likely to be orthogonal.

## Discussion

We studied the host inflammatory response to moderate-severe TBI in two independent multi-centre observational studies of TBI. We found that by using a clustering approach we could identify patients with similar inflammatory mediator profiles that were associated with clinically important outcomes. We sought to identify consistent inflammatory signatures in patients with TBI, as opposed to general polytrauma, and our integrated methods allowed us to explore the clinical features of patients whilst accounting for important confounding variables. By validating a two-biomarker signature our approach was translational instead of simply descriptive.

The strengths of our approach included the delineation of a consistent inflammatory signature in two distinct populations of patients with moderate-severe TBI. We identified analogous early-inflammatory

inflammatory clusters (CENTER-TBI  $n = 106$ , TRACK-TBI  $n = 23$ ). (c) Stacked bar charts showing the relative proportions of patients at each functional outcome level for the GOS-E score for patients in each cluster, in both the discovery and validation cohorts. Although we dichotomised outcomes at a binary level in our analysis (favourable = GOS-E > 4), it is apparent that a greater proportion of patients in the pauc inflammatory cluster had favourable outcomes at all levels of the GOS-E in both cohorts (CENTER-TBI  $p = 0.0036$ , TRACK-TBI  $p = 0.036$ ).

| Immune mediator (pg/ml)    | Discovery cohort              |                              | p value | Validation cohort            |                              | p value |
|----------------------------|-------------------------------|------------------------------|---------|------------------------------|------------------------------|---------|
|                            | Early-inflammatory<br>N = 106 | Pauci-inflammatory<br>N = 29 |         | Early-inflammatory<br>N = 23 | Pauci-inflammatory<br>N = 10 |         |
| IL-15                      | 3.11 (2.59–3.96)              | 1.48 (0.52–2.00)             | <0.0001 | 1.37 (1.05–1.59)             | 0.44 (0.29–0.55)             | <0.0001 |
| IL-6                       | 25 (16–42)                    | 11 (6–24)                    | <0.0001 | 7 (5–32)                     | 1 (0–2)                      | <0.0001 |
| IL-16                      | 257 (186–398)                 | 143 (84–177)                 | <0.0001 | 187 (117–399)                | 1085 (148–2306)              | 0.062   |
| IL-8                       | 24 (19–38)                    | 11 (9–19)                    | <0.0001 | 7 (3–12)                     | 5 (1–97)                     | 0.71    |
| MCP1                       | 324 (216–469)                 | 243 (161–412)                | 0.029   | 45 (30–68)                   | 31 (15–40)                   | 0.047   |
| IL-10                      | 1.8 (0.8–5.7)                 | 1.0 (0.5–2.0)                | 0.0052  | 0.48 (0.30–0.83)             | 0.12 (0.06–0.25)             | 0.0032  |
| TNF $\alpha$               | 0.88 (0.58–1.15)              | 0.67 (0.41–0.87)             | 0.029   | 0.91 (0.71–1.44)             | 0.40 (0.09–0.56)             | 0.0012  |
| IL-13                      | 0.71 (0.34–1.27)              | 1.29 (0.60–2.18)             | 0.032   | 0.36 (0.29–0.58)             | 0.16 (0.03–0.22)             | 0.0011  |
| TNF $\beta$                | 0.09 (0.05–0.18)              | 0.14 (0.13–0.24)             | 0.0071  | 0.26 (0.22–0.28)             | 0.23 (0.18–0.24)             | 0.048   |
| IL-4                       | 0.08 (0.05–0.13)              | 0.10 (0.03–0.14)             | 0.97    | 0.15 (0.08–0.20)             | 0.08 (0.07–0.09)             | 0.0022  |
| IL-7                       | 8.6 (6.8–11.9)                | 4.9 (2.6–7.9)                | <0.0001 | 0.59 (0.39–1.17)             | 0.69 (0.50–1.18)             | 0.40    |
| IL-12p40 (IL23)            | 44 (27–69)                    | 37 (17–63)                   | 0.17    | 20 (9–36)                    | 33 (27–43)                   | 0.12    |
| IL-17a                     | 2.74 (1.93–4.45)              | 3.24 (1.93–5.17)             | 0.47    | 0.74 (0.52–0.92)             | 0.91 (0.79–1.01)             | 0.27    |
| Eotaxin                    | 102 (69–142)                  | 96 (72–109)                  | 0.16    | 48 (28–73)                   | 54 (32–77)                   | 0.95    |
| Eotaxin-3                  | 11 (7–18)                     | 10 (8–14)                    | 0.89    | 3.7 (2.4–5.0)                | 5.9 (3.0–8.2)                | 0.13    |
| MIP1 $\alpha$              | 12 (8–15)                     | 9 (7–12)                     | 0.048   | 6.20 (5.91–6.81)             | 5.67 (5.04–5.94)             | 0.033   |
| MIP1 $\beta$               | 108 (74–162)                  | 90 (78–117)                  | 0.14    | 31 (27–49)                   | 23 (20–34)                   | 0.21    |
| IP10                       | 144 (87–247)                  | 137 (99–179)                 | 0.27    | 27 (10–47)                   | 13 (10–26)                   | 0.38    |
| MCP4                       | 46 (30–61)                    | 52 (36–61)                   | 0.39    | 19 (16–35)                   | 15 (14–22)                   | 0.11    |
| MDC                        | 583 (448–770)                 | 613 (494–784)                | 0.46    | 217 (185–246)                | 256 (234–350)                | 0.038   |
| TARC                       | 137 (95–245)                  | 140 (110–248)                | 0.51    | 18 (12–36)                   | 32 (20–43)                   | 0.16    |
| IFN $\gamma$               | 1.7 (0.9–4.2)                 | 1.2 (0.5–2.7)                | 0.17    | 1.4 (0.3–3.2)                | 0.5 (0.2–1.2)                | 0.12    |
| GM-CSF <sup>a</sup>        | 0.21 (0.14–0.35)              | 0.17 (0.09–0.29)             | 0.12    | –                            | –                            | –       |
| VEGF-A                     | 60 (44–86)                    | 58 (43–98)                   | 0.99    | 41 (19–103)                  | 83 (35–167)                  | 0.22    |
| IL-1 $\alpha$ <sup>a</sup> | –                             | –                            | –       | 0.81 (0.63–1.26)             | 1.19 (0.50–1.78)             | 0.48    |
| IL-1 $\beta$ <sup>a</sup>  | –                             | –                            | –       | 0.11 (0.09–0.17)             | 0.08 (0.06–0.10)             | 0.17    |
| IL-2 <sup>a</sup>          | –                             | –                            | –       | 0.09 (0.05–0.49)             | 0.62 (0.08–1.20)             | 0.020   |
| IL-5 <sup>a</sup>          | –                             | –                            | –       | 0.38 (0.25–0.49)             | 0.30 (0.26–0.32)             | 0.27    |
| IL-12p70 <sup>a</sup>      | –                             | –                            | –       | 0.15 (0.11–0.23)             | 0.06 (0.01–0.09)             | 0.0010  |

All values are presented as median with inter-quartile range. <sup>a</sup>The concentrations of the mediators IL-1 $\alpha$ , IL-1 $\beta$ , IL-2, IL-5 and IL-12p70 the discovery cohort and GM-CSF in the validation cohort were below the lower limit of detection in over 80% of samples, and so these were not included in the statistical analysis between clusters.

**Table 2: Relative inflammatory mediator concentrations in each cluster, across both discovery and validation cohorts.**

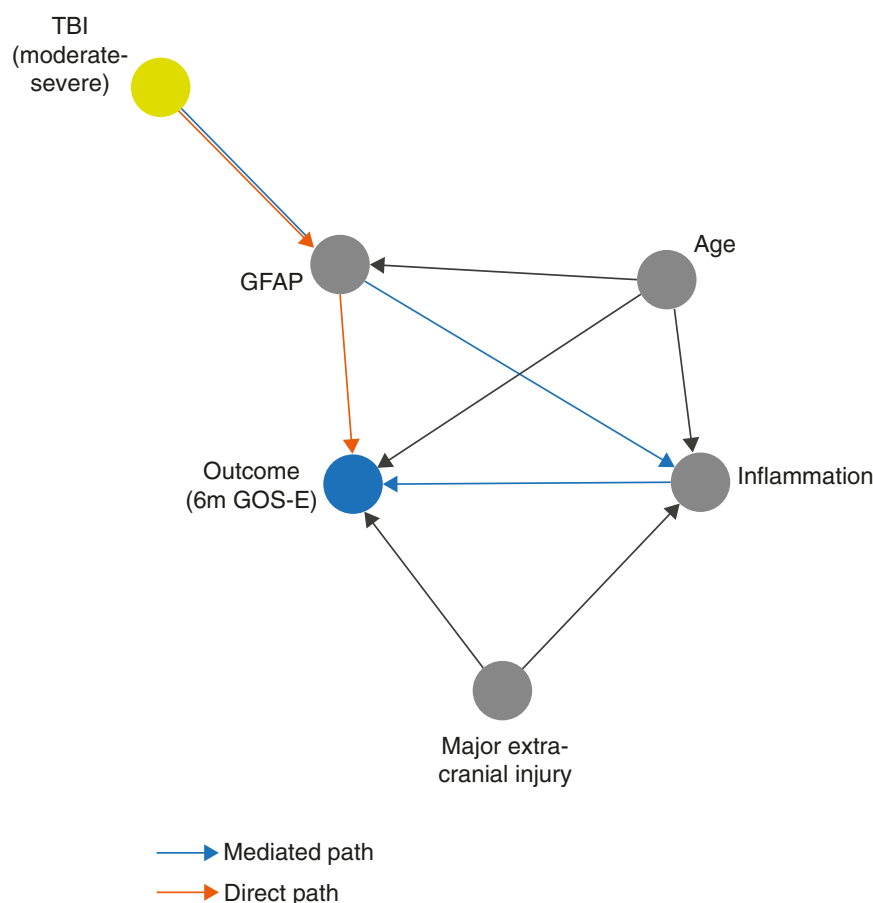
clusters in our validation cohort despite a small sample size. The individual inflammatory mediator concentrations that we used to define our clusters were not associated with unfavourable outcomes in the validation cohort (Supplementary Fig. S1). However, by clustering patients in the same manner as the discovery cohort, we identified patients with an early-inflammatory response who were more likely to have unfavourable outcomes.

There is a general perception that it is difficult to obtain reproducible results when performing multiplex measurements of inflammatory mediators in stored samples, from different studies, with different protocols, measured in different laboratories. The high correlation between the five mediators we used for clustering (Supplementary Fig. S7) meant that by using this enriched set of variables, instead of the entire measured panel, we found similar sub-phenotypes in both cohorts. We were then able to further refine this into a parsimonious signature (IL-15 and MCP1) that

performed well for predicting cluster assignment. Our findings are therefore translational with respect to future patient stratification.

Raised MCP1 (also referred to as chemokine ligand-2; CCL2) in the circulation and cerebrospinal fluid has been associated with severity and outcomes of patients with TBI.<sup>31–33</sup> Persistently raised concentrations of MCP1 in both compartments has been described in longitudinal studies of patients with TBI.<sup>33,34</sup> In a murine TBI model, MCP1 knockout mice (MCP1  $-/-$ ) demonstrated improved neurological recovery and reduced lesion size at 4 weeks compared with their counterparts, which the authors attributed to reduced macrophage recruitment to the lesion site.<sup>35</sup> MCP1 has been shown to play an important role in several brain pathologies including multiple sclerosis, Alzheimer's dementia and ischaemic stroke.<sup>36</sup> A post-mortem study of patients with chronic traumatic encephalopathy reported significant correlations between MCP1 and pTau

|                                                                                                                                                                                                                                                                                       | Discovery cohort              |                              | Odds ratio<br>(95% CI)              | p value | Validation cohort            |                              | Odds ratio<br>(95% CI)               | p value |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|------------------------------|-------------------------------------|---------|------------------------------|------------------------------|--------------------------------------|---------|
|                                                                                                                                                                                                                                                                                       | Early-inflammatory<br>N = 106 | Pauci-inflammatory<br>N = 29 |                                     |         | Early-inflammatory<br>N = 23 | Pauci-inflammatory<br>N = 10 |                                      |         |
| Sex, n (%)                                                                                                                                                                                                                                                                            |                               |                              |                                     |         |                              |                              |                                      |         |
| Male                                                                                                                                                                                                                                                                                  | 90 (85)                       | 20 (69)                      | 2.53 (0.98–6.54)                    | 0.055   | 14 (61)                      | 5 (50)                       | 1.56 (0.25–6.49)                     | 0.56    |
| Age (years), Median (IQR)                                                                                                                                                                                                                                                             | 49 (31–64)                    | 32 (20–56)                   | 0.22 (0.055–0.38) <sup>a</sup>      | 0.0096  | 62 (46–73)                   | 24 (22–30)                   | 0.52 (0.22–0.73) <sup>a</sup>        | 0.0017  |
| Initial GCS, Median (IQR)                                                                                                                                                                                                                                                             | 6 (3–8)                       | 5 (3–8)                      | –0.022 (–0.19 to 0.15) <sup>a</sup> | 0.83    | 3 (3–8)                      | 8 (4–11)                     | –0.37 (–0.63 to –0.022) <sup>a</sup> | 0.038   |
| Initial motor GCS, Median (IQR)                                                                                                                                                                                                                                                       | 3 (1–5)                       | 3 (1–5)                      | 0.0022 (–0.17 to 0.17) <sup>a</sup> | 0.96    | 4 (1–6)                      | 6 (5–6)                      | –0.21 (–0.53 to 0.14)                | 0.23    |
| Baseline pupillary reactivity, n (%)                                                                                                                                                                                                                                                  |                               |                              |                                     |         |                              |                              |                                      |         |
| Both pupils unreactive                                                                                                                                                                                                                                                                | 20 (20)                       | 6 (21)                       | 0.83                                | 0.73    | 5 (24)                       | 2 (22)                       | 1.09                                 | 0.93    |
| Both reactive                                                                                                                                                                                                                                                                         | 68 (69)                       | 17 (61)                      | (0.29–2.40)                         |         | 16 (76)                      | 7 (78)                       | (0.17–7.06)                          |         |
| One pupil unreactive                                                                                                                                                                                                                                                                  | 10 (10)                       | 5 (18)                       |                                     |         | –                            | –                            |                                      |         |
| Unknown                                                                                                                                                                                                                                                                               | 8                             | 1                            |                                     |         | 2                            | 1                            |                                      |         |
| Pre-hospital/ED hypotension, n (%)                                                                                                                                                                                                                                                    |                               |                              |                                     |         |                              |                              |                                      |         |
| Definite                                                                                                                                                                                                                                                                              | 10 (11)                       | 2 (8.7)                      | 1.33                                | 0.73    | 4 (17)                       | 0                            | NA <sup>b</sup>                      | 0.99    |
| No                                                                                                                                                                                                                                                                                    | 79 (85)                       | 21 (91)                      | (0.27–6.53)                         |         | 19 (83)                      | 10 (100)                     | (0–∞)                                |         |
| Suspected                                                                                                                                                                                                                                                                             | 4 (4.3)                       | 0 (0)                        |                                     |         | –                            | –                            |                                      |         |
| Unknown                                                                                                                                                                                                                                                                               | 13                            | 6                            |                                     |         | –                            | –                            |                                      |         |
| Pre-hospital/ED hypoxia, n (%)                                                                                                                                                                                                                                                        |                               |                              |                                     |         |                              |                              |                                      |         |
| Definite                                                                                                                                                                                                                                                                              | 4 (4.4)                       | 4 (15)                       | 0.26                                | 0.071   | 7 (30)                       | 0 (0)                        | 0.41                                 | 0.54    |
| No                                                                                                                                                                                                                                                                                    | 81 (90)                       | 21 (81)                      | (0.060–1.12)                        |         | 1 (4.3)                      | 1 (10)                       | (0.02–7.28)                          |         |
| Suspected                                                                                                                                                                                                                                                                             | 5 (5.6)                       | 1 (3.8)                      |                                     |         | –                            | –                            |                                      |         |
| Unknown                                                                                                                                                                                                                                                                               | 16                            | 3                            |                                     |         | –                            | –                            |                                      |         |
| Marshall CT score, n (%)                                                                                                                                                                                                                                                              |                               |                              |                                     | 0.85    |                              |                              |                                      | 0.066   |
| I                                                                                                                                                                                                                                                                                     | 4 (4)                         | 1 (3.6)                      | –                                   |         | 3 (13)                       | 4 (40)                       | –                                    |         |
| II–IV                                                                                                                                                                                                                                                                                 | 41 (41)                       | 13 (46)                      | 0.79 (0.081–7.70)                   |         | 13 (57)                      | 6 (60)                       | 2.89 (0.49–17.2)                     |         |
| V–VI                                                                                                                                                                                                                                                                                  | 56 (55)                       | 14 (50)                      | 1.00 (0.10–9.66)                    |         | 7 (30)                       | 0 (0)                        | NA <sup>b</sup> (0–∞)                |         |
| Unknown                                                                                                                                                                                                                                                                               | 5                             | 1                            |                                     |         | –                            | –                            |                                      |         |
| Injury severity score, Median (IQR)                                                                                                                                                                                                                                                   | 34 (26–50)                    | 29 (25–41)                   | 0.11 (–0.059 to 0.27) <sup>a</sup>  | 0.20    | 25 (17–28)                   | 22 (12–28)                   | 0.17 (–0.18 to 0.49) <sup>a</sup>    | 0.60    |
| Major extracranial injury, n (%)                                                                                                                                                                                                                                                      | 71 (67)                       | 15 (52)                      | 0.52 (0.23–1.22)                    | 0.13    | 6 (33)                       | 4 (44)                       | 0.63                                 | 0.57    |
| <b>Outcomes</b>                                                                                                                                                                                                                                                                       |                               |                              |                                     |         |                              |                              | (0.12–3.22)                          |         |
| GOS-E at 6 m, n (%)                                                                                                                                                                                                                                                                   |                               |                              |                                     |         |                              |                              |                                      |         |
| Favourable                                                                                                                                                                                                                                                                            | 31 (33)                       | 18 (64)                      |                                     |         | 8 (38%)                      | 7 (88%)                      |                                      |         |
| Unfavourable                                                                                                                                                                                                                                                                          | 64 (67)                       | 10 (36)                      | 3.72                                | 0.0036  | 13 (59%)                     | 1 (12%)                      | 11.38                                | 0.036   |
| Unknown                                                                                                                                                                                                                                                                               | 11                            | 1                            | (1.53–8.99)                         |         | 2                            | 2                            | (1.17–110.42)                        |         |
| Patients in the early-inflammatory clusters were associated older and more likely to have worse outcomes in both cohorts. <sup>a</sup> Correlation coefficient (r) for continuous variables. <sup>b</sup> Impossibly large estimate (OR >2 × 10 <sup>7</sup> ) and so values omitted. |                               |                              |                                     |         |                              |                              |                                      |         |
| <b>Table 3: Association between baseline clinical features and outcomes of patients assigned to each cluster in both cohorts.</b>                                                                                                                                                     |                               |                              |                                     |         |                              |                              |                                      |         |



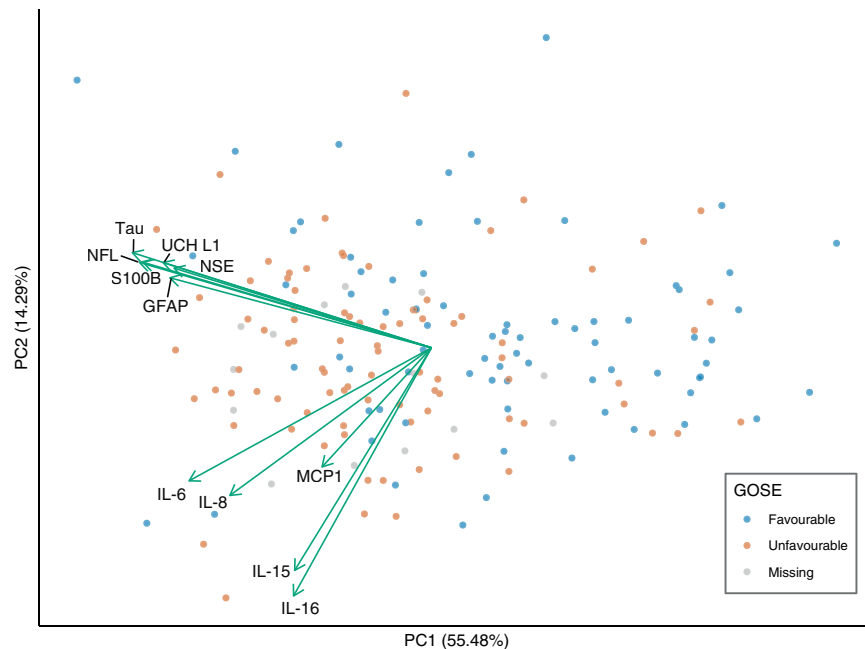
**Fig. 4:** Directed acyclic graph demonstrating the causal model used to describe the relationships between TBI, outcome at 6 months and inflammation whilst accounting for important confounders such as age and major extracranial injury (MEI). Age was found to confound both inflammation and GFAP with respect to outcome, whilst MEI was conditionally independent of GFAP concentrations. The orange and blue lines demonstrate the direct and mediated causal paths respectively. Mediation analysis was used to calculate the contribution of inflammatory processes on patient outcomes.

staining, burden of repetitive head injury, and reactive microglial (Iba1+) cell density.<sup>37</sup>

Additional strengths of our study include both cohorts being multi-centre and the clinical and biological similarities between the clusters. For example, both early-inflammatory clusters were associated with increasing age. Although aging is associated with chronic low-level inflammation and increased risk of auto-immunity,<sup>38</sup> it is not consistently associated with excessive innate immune activation compared with younger patients in critical illness. Older patients with sepsis have higher circulating concentrations of C-reactive protein (CRP) and matrix-metalloproteinase-8 (MMP-8) but not IL-6, IL-8 or IL-10, and their transcriptomic profiles are associated with suppressed cytokine and toll-like receptor activation.<sup>39</sup> Others have not found evidence of greater innate immune activation in older patients with sepsis.<sup>40</sup> Contrasting findings have been observed in patients with acute respiratory distress

syndrome where age is associated with higher concentrations of IL-6, p-selectin and myeloperoxidase (MPO) in bronchoalveolar fluid,<sup>41</sup> but not in the circulation.<sup>42</sup> Together these results suggest that excessive innate inflammatory activation is not a consistent feature of all older, critically unwell patients.

To disentangle the relationship between age and the inflammatory response to TBI we used a mediation analysis approach (Fig. 4, [Supplementary Table S5](#)). This suggested that the inflammatory component mediated the association between TBI and outcome when age was accounted for. Interestingly, this mediated effect was not observed when patient age was not included as a confounder ([Supplementary Table S6](#)). Nor did we find a direct relationship between age and the first PC of the inflammatory response, raising the possibility that inflammation potentiates the influence of age on the outcome of TBI. This implies that older brains may be more susceptible to the effects of the



**Fig. 5:** Principal component analysis loadings plot of the brain injury biomarkers (GFAP, NFL, NSE, S100B, Tau, UCH L1) and inflammatory mediators (IL-6, IL-8, IL-15, IL-16, MCP1) measured in patients recruited to CENTER-TBI ( $n = 135$ ). Brain injury biomarkers were colinear with each other, as were the inflammatory mediators that defined the early-inflammatory cluster. However, as two different sets of protein biomarkers they appear to be orthogonal, primarily separated in the second principal component (PC). To assess whether they were orthogonal across all principal components, we calculated the dot product of the brain injury biomarker and inflammatory mediator loadings, weighted by their relative explained variances. The calculated dot product was equal to  $-0.019$  (95% CI  $-0.074$  to  $+0.035$ ) which was consistent with an orthogonal relationship across all PCs.

immune response to TBI—in keeping with the suggestion that the relationship between age and inflammation integrates both a dysregulated immune response, and an increased end-organ susceptibility to this response which has been referred to in the literature as “inflammaging”.<sup>43</sup>

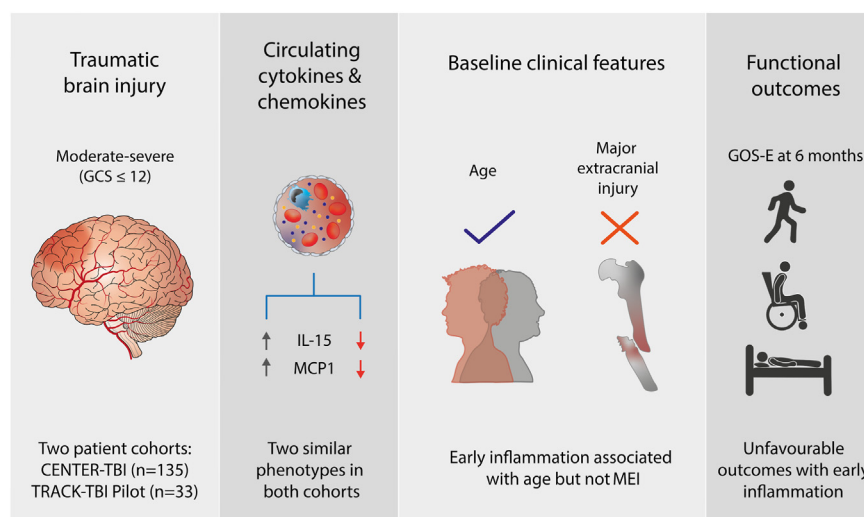
Our finding that the brain injury and inflammatory mediators were orthogonal in the principal component space demonstrated how measuring inflammatory mediators might add predictive power that might not otherwise be captured (Fig. 5). It also supported the hypothesis that patient outcomes were dependent on two different mechanisms: primary injury and secondary injury from inflammatory damage, which is a modifiable component from a therapeutic perspective.

The association between age and aberrant inflammatory response to TBI that we observed is consistent with data indicating a distinct activation profile in peripheral blood mononuclear cells (PBMC) acutely (<48 h) after TBI in young vs elderly patients. In the elderly, both CD4 and CD8 T cell activation occurred, whilst young patients with TBI exhibited an increase in CD4 cells only.<sup>44</sup> This is further supported by experimental data in mice, where aged TBI mice exhibited poorer functional outcomes and had a dysregulated

systemic, meningeal and brain tissue immune response compared to young mice.<sup>45</sup>

Patients in the identified clusters had other notable clinical characteristics, particularly the absence of a significant association with ISS or major extracranial injuries. Both of these factors are important confounders when evaluating the inflammatory response and functional outcomes of patients. This provides strong evidence that the magnitude of the early inflammatory response (and allocation into mechanistic clusters) is not simply due to a greater injury burden but may instead indicate endogenous responses. These clinical findings and attributes of patients in each cluster are summarised in Fig. 6.

The measurement of inflammatory mediators at the day 2/3 interval after injury in the discovery cohort showed that inflammatory clusters are relatively stable, but that the most predictive status associated with outcomes was cluster assignment on day 1. This is consistent with other studies of the inflammatory response in traumatic injury.<sup>46,47</sup> Cabrera and colleagues showed that transcriptional responses in the hyper-acute, 2-h window after injury were the most predictive of multi-organ dysfunction syndrome (MODS) and that these early inflammatory signals were transient and



**Fig. 6:** Visual summary of the study findings, highlighting the patient population that was studied, key components that defined the inflammatory endotypes and their relationship with confounding variables (age, major extracranial injury) and patient outcomes.

subsided after 24 h.<sup>47</sup> Other groups have described the importance of IL-17 and Th17 cells in the immune response following trauma, however these signals were most reliably detected several days after the initial injury.<sup>48,49</sup> The clinical implication of this hyper-acute decaying, dynamic process is that patient stratification will have to take place at an early stage in their care to facilitate the stratification of therapy in future trials.

Critically, the implementation of such endotypic stratification in trials of inflammatory modulators may not need measurement of all five mediators that were included in our analysis, since we demonstrated that the combination of IL-15 and MCP1 provided a parsimonious, but still effective basis for stratification.

### Limitations of the study

There are several important limitations to our study. Given the multi-centre nature of our data there were significant differences in several baseline characteristics between patients in each cohort that include proportion of males, initial motor component of the GCS, ISS, proportion of patients with MEI and Marshall scores of CT images (Table 1).

We recognise that the validation cohort was of a relatively small size. In part, this was due to the inclusion criteria we applied (particularly injury severity; Fig. 1). This would explain why the associations we found between endotypes and outcomes had wide confidence bounds when calculating odds ratios and might be consistent with a sparse data bias.<sup>50</sup> In addition, due to the nature of patient recruitment in the discovery cohort we could not account for unexplained confounding factors in our causal model which is an important limitation of our mediation analysis. However, the inflammatory mediator analyses that we

undertook will, at a later stage, be applied to larger cohorts of patients within and outside these studies to demonstrate a relationship between the early systemic inflammatory response and prolonged neuroinflammation.

The homogeneity between the clusters in each cohort was not always consistent; the correlations between IL-16 and the other cytokines were different in the validation and discovery cohorts (Supplementary Fig. S8). Patients in the validation cohort with the early-inflammatory phenotype had lower baseline GCS. In addition, the confidence bounds of the association between early-inflammation and outcome were wide. These differences could be partly explained by the different blood fractions that the inflammatory mediators were measured in (Discovery: serum, Validation: plasma), and differences in the times at which samples may have been obtained, especially if there were delays in transferring patients to recruitment centres. The multiplex MSD V-plex assays are validated to measure immune mediators in both serum and plasma and we did not make direct comparisons of mediators between cohorts because of these differences in sample collection. We addressed this limitation by clustering separately in each cohort and by defining a parsimonious signature across both cohorts. Additionally, some of the other limitations might be addressed by a larger validation cohort.

Of interest is the relatively large size of the early-inflammatory clusters in each cohort, which suggests that early activation of inflammatory mechanisms in patients with moderate-severe TBI is common, and therefore may be an important therapeutic target in patients. However, the labels that we have provided for these sub-phenotypes are likely to be over-

simplifications of biological processes that will require more detailed characterisation in mechanistic studies. It does, however, highlight the importance of a stratified approach to treatment, as immunomodulation might be a poor treatment strategy in patients with the pauc-inflammatory phenotype.

In conclusion, we have shown that a small number of inflammatory mediators can be used to define inflammatory clusters in patients with TBI that are associated with outcomes and this approach performed well to identify similar patients in an independent cohort.

#### Contributors

Conceptualization: DKM, JCS, OT, GTM, EN, ERZ.

Methodology: RJS, ACC, EN, JKY.

Validation: GTM, JKY, KKWW, FK, RJS, JPP, OT.

Data curation: RJS, ACC, JKY.

Formal analysis: RJS, ACC, EN, DKM.

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Project administration: DKM, AIRM, GTM, JCS.

Funding acquisition: DKM, AIRM, GTM.

Contributors from the CENTER-TBI and TRACK-TBI were involved in the design of the study, sample and data collection.

First authorship order determined by the relative contributions of authors to the formal analysis (RJS: discovery and validation, ACC: discovery, EN: discovery). All authors read and reviewed the final version of the manuscript.

#### Data sharing statement

Data access is conditional to an approved study proposal; there are no end dates to the availability. The CENTER-TBI and TRACK-TBI Pilot data used in this study are available to researchers who provide a methodologically sound study proposal that is approved by the CENTER-TBI and TRACK-TBI management committees. Proposals may be submitted online to CENTER-TBI (<https://www.center-tbi.eu/data>). A data access agreement is required, and all access must comply with regulatory restrictions imposed on the original study. No patient-identifiable information is made available, and all data have been anonymised.

#### Declaration of interests

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A list of study consortia members is available in the [Supplementary Material](#).

#### Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ebiom.2024.105310>.

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